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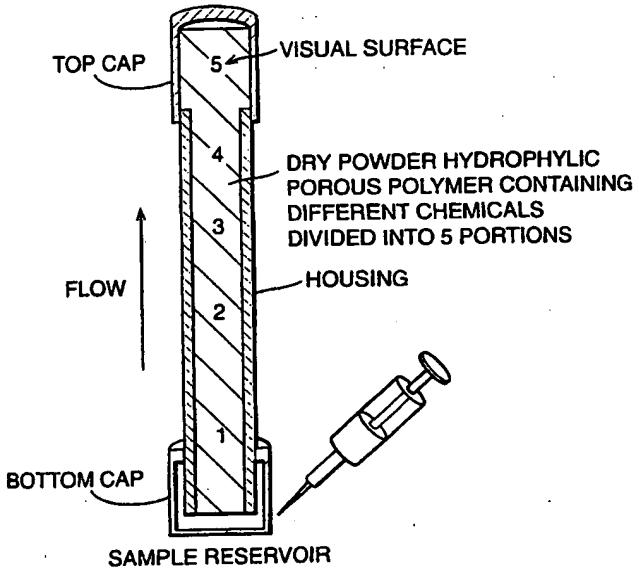

(57) Abstract

A solid phase apparatus, for detection of endotoxin in a liquid sample using reagents located within solid support material, comprises endotoxin sensitive reagent and buffer components. When a liquid sample containing endotoxin is applied to the apparatus the endotoxin sensitive reagent reacts with the endotoxin to form at least one product, and following contact of the at least one product with buffer components, an endotoxin indicator is formed. Also disclosed are a method and a kit to test for endotoxin.

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SOLID PHASE TEST FOR ENDOTOXIN

This invention relates to a solid phase test for endotoxin, in particular a test such as in a flat sheet or in capillary form for the detection of endotoxin in aqueous solutions. More particularly the invention relates to a direct solid phase chromogenic assay, which permits a quick biochemical identification of endotoxin and is sensitive to picogram endotoxin amounts.

There is a high demand for testing for contamination of solutions and biological fluids in medicine and biotechnologies; for instance, the quality of water used for the dilution of solute concentrates for hemodialysis. Specifically, there is a need for pyrogen free water to make dialysate, and for monitoring of the dialysate in the dialysis machine itself in centres or at home or other non-laboratory settings. In this respect, the production of sterile and pyrogen free dialysate needs a pyrogen control method which will give a "yes" or "contaminated" result. Endotoxin, a potent pyrogen specifically from gram-negative bacterial cell wall components, is one of the most known key contaminants. Measurements of endotoxin are today used as the major safety parameter of infusions and fluids in general, drugs and devices, in the pharmacology and food industry. The most available method to qualitatively and quantitatively detect endotoxin in biological or aqueous solutions is based on the limulus amoebocyte lysate (LAL) test known in the art.

The LAL test was discovered in 1964 by Levin and Bang[1]. Horseshoe crab (limulus) amoebocyte lysate provokes together with endotoxin from gram-negative bacteria a gelatination induced by a stepwise activation of several coagulation factors contained in the lysate. An assay comprising the extract (lysate) of horseshoe crab blood cell (amoebocyte) to induce this gelatination in a reagent glass as a liquid phase system is well known as a method and it is called the limulus test. The application of the LAL to measure endotoxin involves the gelatination of the sample (gelation method, turbidimetry assay) in the reagent glass or a more sensitive method based on a chromogenic substrate and measurement by optical absorption. Today different LAL from horseshoe crab [2] are

commercialized through the world: *Limulus Polyphemus*, *Tchypleus tridendatus*, *Tachypleus gigas* and *Carcinoscorpius rotundicauda*.

All commercialized LAL testing methods based on a chromogenic substrate are liquid-phase assays. The principle is that in a reagent glass containing a lysate and sample, bacterial endotoxin contained in the sample initiates activation of a cascade of serine protease (Factor B, Factor C, Proclotting enzyme, Coagulin) enzymes in LAL that cleave the end product coagulogen into a peptide coagulin which produces clotting. The use of a synthetic chromogenic or fluorogenic substrate increases the sensitivity of the testing and makes the quantification of endotoxin possible. As described by Iwanaga et al[3], a synthetic peptide is used having an amino acid sequence in common with the hydrolysis sites of coagulogen, namely the chromogenic substrate Boc-Leu-Gly-Arg-p-nitroanilide (pNA) or the fluoro-genic substrate Boc-Leu-Gly-Arg-4-methylcoumaryl-7-amide. In the presence of LAL and endotoxin, the colourless substrate is rapidly cleaved to form the chromophore p-nitroaniline or the fluorogenic 4-methylcoumaryl-7-amide.

There are today increasing improvements in the accuracy and sensitivity of liquid phase assays. In US patent 5 605 806, S. Tanaka and H. Tamura proposed a LAL and an antibody to neutralize the false positive results due to (1 ->3)-ß-glucan. Shigenori et al proposed the use of factor G activation to resolve the same problem (US patent 5,641,643). Besides the liquid phase reaction systems, a method for lysate immobilization to wells of a polystyrene microplate has been published. In US patent 5,550,30, Tanaka disclosed a quantitative kit for endotoxin determination which comprises an insoluble carrier on which is immobilized an endotoxin-sensitive factor derived from a limulus amoebocyte which specifically reacts with endotoxin without reacting with ß-glucan. The test system involves the indication of endotoxin when colouring occurs in the liquid phase.

Quick and simple testing for endotoxin is important in a number of circumstances, including when changing the dialysis machine, or monitoring tap water. It is also desirable to determine endotoxin levels of the fluids in a laboratory-setting. The

degree of contamination of water and dialysate fluids falls into several categories, but in all cases it is desirable to monitor endotoxin present in the fluid on a frequent basis. A simple and quick method for determining qualitatively endotoxin in a test solution is thus much sought in the art.

The present invention provides an analytical test strip for the detection of endotoxin in aqueous solutions in which the strip is wetted at the bottom with the sample. After a certain period of time, until the sample flows from the bottom to the top, an indicator is added at the top, to develop a red colour which is indicative for the presence or the absence of endotoxin in the sample.

Using a technique of a solid phase chromogenic substrate assay, the present invention involves an improvement to a quick test strip for the detection of endotoxin. The test strip provides a dry analytical element for endotoxin which comprises an absorbent carrier divided in four portions impregnated with: 1) a reagent system of limulus factors; 2) synthetic chromogenic substrate derivatized with p-nitroaniline; 3) a buffer system containing chloride acid and sodium nitrite; 4) a buffer system containing chloride acid and ammonium. The test also includes a solution containing N1-naphtylethylenediamine which is added at the top of the test trip to develop a coloration by coupling the derivatized p-NA to N1-napthylethylenediamine as positive results.

The present invention provides apparatus and method as defined in the claims, in particular an analysis method comprising applying a aqueous sample solution to the bottom of the described dry analytical element to react with endotoxin, and determining coloration on the top of the dry element by coupling N1-napthylethylenediamine. The present invention also provides an endotoxin kit using the above described dry analytical element.

A further object of this invention is to provide a method for preparing the above mentioned dry analytical element and/or the portions of the dry analytical element for preparing the above described endotoxin test strip.

The method involved in the invention is a direct solid phase chromogenic calorimetrical detection. Four supports are arranged successively in the same alignment to achieve a flow communication with each other. The supports are mounted in a closed and pyrogen free casting module. The test strip comprises a solid support having four portions (at least three portions) being in flow communication with each other whereby reaction products can flow from one portion to the next. The solid support is preferably shaped in the form of strip, with the first, second, third and fourth portions being arranged on the strip in the same plane. The sizes of the portion may be the same or different. The portions are manufactured to absorb the different chemicals and dried, and attached on the strip in a way that flow communication occurs from one to the next.

The test strip would be submerged with the sample at the bottom to wet the first portion and allow liquid to flow from bottom to the top of the test strip. The sample solution would start to flow by capillary action from one portion to the next higher portion. Thus, the reactants occurring in each portion would be transported by capillary flow to the last portion to develop a red coloration as a positive result.

The first portion contains a solid support and a limulus amoebocyte lysate or substances containing endotoxin sensitive factors which react with endotoxin to produce coagulin. The method of the immobilization of the lysate or of the endotoxin sensitive factor to the solid support are: 1) absorption; 2) adsorption by ionic forces or/and hydrophobic interaction, 3) covalent binding. The procedures for binding the lysate in the solid support are generally known the art. The lysate may be the commercial available limulus amoebocyte lysate such as *Limulus Polyphemus*, *Tchypleus tridendatus*, *Tachypleus gigas* and *Carcinoscorpius rotundicauda*. Preferably a lysate without Factor G may used in order to avoid a positive false reaction with (1-3)-ß-D-Glucan contained in the sample or in the solid support. A purified form of a mixture containing factors C, B and proclotting enzymes, which react with endotoxin to produce coagulin may also be used. Preferably a lysate preparation known in the art without Factor G which does not react with (1-3)-ß-D-Glucan may also be used. The amount and biological activity

of endotoxin reagents, and the material and size of the solid support determine the sensitivity of the test strip. Upon wetting of the solid support with the sample, sample should react with immobilized lysate or substances containing endotoxin factors to activate Factor and clotting enzymes. Depending upon the presence of endotoxin in the samples, endotoxin activates the bound lysate in the following reaction steps:

(1) Endotoxin -> activated Factor C

(2) activated Factor C -> Factor B -> activated Factor B

(3) activated Factor B -> Proclotting enzyme

The clotting enzyme produced through the cascade reaction by the action of activated Factor C or proclotting enzymes is capable of hydrolysing coagulin or an amide linkage of a synthetic peptide substrate at the specific sites intermediate between Arg and Gly or intermediates between Arg and Thr.

The solid support which is employed in this test strip is one which is capable of absorbing endotoxin from the sample. When wetted, the sample containing endotoxin reacts with reagents in the first portion and the reaction products are transported by flow to the dry region of the first support and thus to the second portion of the test strip. In addition, the solid support is one which is capable of absorbing both sample and binder. In order to achieve a flow communication between the portion, it is preferable to use an hydrophillic polymers solid support. Examples of suitable supports are chromatographic papers, nitrocellulose, dextran and glass fibres. Preferably, chromatographic hydrophobic polymers are used though if using chromatography papers, it is important to use reagents which do not react with ß-glucan, as described for example in US patent 5,550,030.

The second portion contains a solid support and a synthetic substrate. A typical substrate to be immobilized in the second portion is t-butoxycarbonyl-leucyl-glycyl-

arginine-paranitroanilide (Boc-Leu-Gly-Arg-pNA) to release paranitroanilide. Usable synthetic peptides with a known detectable group are those described in EP-A-0000063, EP-A-0018112, WO79/00602, WO82/02382 and US 4,188,264. However, synthetic peptide substrates for activated Factor C as well as synthetic peptide substrates for activated clotting enzymes in which a carboxyl group of the C-terminated arginine is substituted with the colour-developing residue p-nitroaniline, p-(N,N-diethylamine)aniline, p-(N-ethyl-N-ß-hydroxyethyl) aniline can also be used. Possible methods of immobilization of the chromogenic substrate to the solid support are: 1) absorption; 2) adsorption by ionic forces or/and hydrophobic interaction, and 3) covalent binding. Preferably, the support is absorbed with the substrate. The solid support is typically made with the same material as the first support and it is one which is capable of absorbing the liquid and reactants flowing from the first support, and which when wetted is this way, provides for flow and analyte by capillary attraction from the first portion, and through the second portion into the third portion. Upon contact with the liquid and reactants flowing from the first support, solution should react with immobilized chromogenic substrate. Like the first portion, examples of suitable supports are chromatographic papers, nitrocellulose, dextran and glass fibres. Preferably, chromatographic papers are used. The solid support absorbed with the chromogenic substrate is one which is capable of absorbing reactants flowing from the first support. When wetted, the so-called reactants are capable of reacting with the second portion and the reactants with the new reaction products being transported to the dry regions and thus to the third portion of the test strip. The solid support is so manufactured (with attention to the amount of absorbed synthetic chromogenic substrate, size of the portion, material of the solid support) that activated clotting enzyme and/or factor B flowing from the lower portion reacts with substrate in the second portion and the chromophore p-nitroaniline is cleaved. The cleaved p-nitroaniline is then transported to the next portion.

The third portion contains a solid support and a buffer containing 1 N HCl and 0.1% (w/v) sodium nitrite. Preferably, to make the support; it is impregnated with the buffer system, dried and is made with the same material as the second support.

When dried, the solid support is also one which is capable of absorbing the liquid and the chromophore p-nitroaniline flowing from the second support, and which when wetted is this way, provides for flow from the second portion, and through the third portion into the fourth portion.

The fourth portion contains a solid support and a buffer containing N HCl containing 0.5% ammonium. Preferably, to make the support, it is impregnated with the buffer system, and dried. When absorbed with the buffer system and dried, the solid support is also one which is capable of absorbing the liquid and the chromophore p-nitroaniline flowing from the third support, and which when wetted is this way, provides for flow from the third portion, and through the dried region.

The third and fourth portions are those through which when the flowing free p-nitroaniline released by the action of enzymes is derivatized to its diazanium salt, typically formed in the fourth portion.

The fifth portion is a neutral portion in which a drop of 0.05% N1-naphtylethylenediamine in 40-50% (v/v) is added to form a highly visible red colour by coupling of the derivatized p-NA to N1-napthylethylenediamine.

The test strip described in this invention can suitably be prepared from any matrix material through which an aqueous solution can flow by capillarity. Suitable materials for chromatographic strips are paper, nitrocellulose and hydrophillic polymers. Because of the presence of ß-glucan in cellulosic material, paper is not usually suitable without special treatment as it induces false positive results. In this invention, it is desirable to use hydrophillic polymers which allow vertical capillary flow excluding a bibulous lateral flow such as polyethylene sheet material.

There now follows description of specific embodiments of the present invention, illustrated by drawings in which:-

Fig. 1 shows a schematic representation of a capillary test strip;

Fig. 2 shows a schematic representation of a paper test strip;

Fig. 3 shows mode of operation of a test strip;

Fig. 4 shows variation of substrate sensitivity and resulting lysate activity; and

Fig. 5 shows a quantitative test strip.

Example 1: capillary test strip

As illustrated in figure 1, a glass howl capillary glass with a flat side, an internal dimension of 0.25 cm and an external dimension of 0.28 cm and a length of 4 cm has been used as the casting module. For the bottom of the capillary there is a cap which is used as the sample reservoir, whereas a second cap closes the top of the capillary and permits entry of chemicals for the colour reaction. The module is constructed in a way that it remains pyrogen free. The howl module is successively filled with dried hydrophillic porous spherical material (200-500 μm). The hydrophillic porous polymer is in separate portions, impregnated as follows:

Portion 1: hydrophillic polymeric powder was incubated under pyrogen free conditions in 0.5 IU/ml (1 g/ml) of limulus amoebocyte lysate (Charles River- Sulzfel Germany) until saturated absorption of the liquid was reached. After drying, the module has been filled to reach a height of 1 cm. 0.1 cm has then been filled with neutral polymeric powder.

Portion 2: 10 μmol of chromogenic substrate (t-butoxycarbonyl-leucyl-glycyl-arginine-paranitroanilide) for endoxin (Pefachrome LAL Code NR 11179-01 ISO 9001-1N29001 Pentapharm AG BASEL CH) was diluted in 6.6 ml pyrogen free water. 1g of hydrophillic polymeric material was also incubated with 1 ml until saturated absorption was reached. After drying the module has been filled to reach a height of 0.5 cm. 0.1 cm has then been filled with neutral polymeric powder.

Between portions 2 and 3: Tris-HCl is mixed with polymeric powder to obtain a concentration of 0.025 mmol/l and inserted between portions 2 and 3 with a height of 0.5 cm. 0.1 cm has been filled with neutral polymeric powder.

Portion 3: Polymeric powder was submerged in a solution of 1 N HCl containing 0.1 % (w/v) sodium nitrite, and dried.. A quantity sufficient for 0.5 cm has been inserted into the module. 0.1 cm has been filled with neutral polymeric powder.

Portion 4: 1 cm of polymeric powder was submerged with in solution of 1 N HCl containing 0.5 % (W/v) ammonium sulfate, dried and has been filled into the module. 0.1 cm has been filled with neutral polymeric powder.

Portion 5 : 2.5 mg of N1-naphthlethylenediamine in mixed with 0.5 cm polymeric material.

Example 2: paper test strip

The strip can prepared from any matrix material through which the test fluid can vertically flow by capillarity; as illustrated in figure 2, a polystyrene dipstick was constructed to be fitted in a sterile bottle. The active surface of the test strip was 0.5 cm x 4.2 cm, separated every 0.5 cm between the first portion and the third portion with 0.5 cm x 0.2cm dry Sephadex. The active surface between the sephadex strip was coated with Scotch adhesive transfer tape # 969 (3M, USA, 55144)

Portion 1: consisted of 0.5cm x 0.5 cm ultra high molecular weight polyethylene sheet material (Porex Technologies) which was incubated for 10 min under pyrogen free conditions in 0.5 IU/ml (1 g/ml) of limulus amoebocyte lysate (Charles River-Sulzfel Germany). After drying, it was applied to the bottom of the test strip. A neutral 0.5 cm x 0.1 cm portion was added above the neutral sephadex.

Portion 2: consisted of 0.5cm x 0.5 cm ultra high molecular weight polyethylene

sheet material (Porex Technologies) which was spotted with 1.5 μmol/ml of chromogenic substrate (t-butoxycarbonyl-leucyl-glycyl-arginine-pa-ranitroanilide) for endoxin (Pefachrome LAL Code NR 11179-01 ISO 9001-1N29001 Pentapharm AG BASEL CH). The strip was applied just above the neutral sephadex portion.

Between portions 2 and 3: consisted of 0.5cm x 0.5 cm sephadex and Tris-HCl at a concentration of 0.025 mmol/l.

Portion 3: consisted of a 0.5 cm x 0.5 cm strip of filter paper (Schleicher & Shuel 23 SL) which was submerged with solution of 1 N HCl containing 0.1 % (w/v) sodium nitrite, dried and applied above the neutral sephadex portion.

Portion 4: consisted of a 0.5 cmx 0.5 cm strip of filter paper (Schleicher & Shuel 23 SL) which was submerged with in solution of 1 N HCl containing 0.5 % (W/v) ammonium sulfate, dried and applied above the third portion.

Portion 5 : consisted of a 0.5 cmx 0.5 cm strip of filter paper (Schleicher & Shuel 23 SL) applied above the fourth portion.

For both examples the assay is carried out as follows:

1) add the sample to the first portion of the test strip or immerse the first portion in the sample (example 2) or fill the reservoir with the sample (example 1).

2) heat the test strip to 37 °C device to activate the reaction;

3) allow sufficient time (such as 4 min) for the sample to flow from the bottom to the top;

4) dry the test strip by removing the sample reservoir from the bottom and opening the bottom of the casting; and

5) for colour development, a drop of 47.5% ethanol is added, coloration develops after drying in air.

The chemical reactions involved in each portion are described in Figure 3. In

portion 1, depending upon the presence of endotoxin in the samples, clotting enzyme is produced through the cascade reaction by the action of activated Factor C or proclotting enzymes. In portion 3 after the capillary flow through the chromogenic substrate and in the presence of Tris-HCl, the activated clotting enzyme and/or factor B flowing from the lower portion cleaves p-nitroaniline. The cleaved p-nitroaniline being transported to the third and fourth portion and derivatized to its diazonium salt. In the fifth portion, the diazonium salt is coupled to N1-napthylethylenediamine to form a diazoamino of a red colour.

The assay can be performed with different sensitivities, according to the grade of contamination to be analyzed. In the special application to test solutions used in hemodialysis therapy and according to Pharmacopoeia prescriptions, several test strips have been elaborated to read the minimal acceptable concentration of endotoxin in aqueous solution (Table 1).

Table 1

Applications	Substitution solution for Hemodiafiltration	Hemodialysis water	Dialysate solution	Water for on-line HDF
Lowest LPS concentration in EU/ml	0.05	0.25	0.5	1

In respect to the sensitivity of the test, numerous modification can be performed, such as by varying the titration of the LAL-concentration and the matrix material used in the first portion. By the application of high molecular weight polyethylene sheet material (Porex Technologies) and using several titrations, of limulus amoebocyte lysate varying from 0.1 to 0.5 IU/ml and several matrices, for examples 1 and 2, it is possible to prepare 3 classes of lysate concentrations. The method used to prepare the desired lysate concentration is first based on a liquid chromogenic substrate assay in a microplate according to the LAL-test Endochrome-K™ (US. License No 1073) except that the LAL-concentration is not related to the protocol. Figure 4 illustrates the resulting absorbance due to the

variation of the LAL which allow 4 classes of test-strips. Applied to the test-strip of example 2, it is possible to prepare a test-strip especially for the detection of endotoxin concentration in dialysate-solution, for which the acceptable limit is prescribed to be below 0.5 EU/ml. Detection spots for this prepared test strip are illustrated in Figure 5.

References:

1 J. Levin and F.B. Bang Bull. Johns Hopkins Hospital, 115, 265-274 (1964)

2 Progress in Clinical and Biological Research, Volume 93; "Endotoxin and their Detection with the Limulus Amoebocyte Lysate Test"; edited by Stanly W. Watson, Jack Levin and Thomas J. Novitsky (1982); pages 7-24

3 Iwanaga et al. Hemostasis, Z, 183-199 (1978)

4 J. Clin Microbiol. 17, 1050-1053 (1983)

Related Application Data:

1 Solid phase chromatographic immuno assay- Rosenstein-US patent 5,591,645 (Jan 7, 1997)

2 Method for the detection of protein urine- Cahil et al: US patent 5,593,895 (Jan 14, 1997)

3 Reagent for endotoxin-specific assay- Tanak et al, US patent 5,550,030 (Aug 27 1996)

4 Test strip for blood glucose testing- Kelvin J. Philipps- US patent 5,556,761 (Sep 17, 1996)

5 Quantitative detection of analytes on immunochromatographic strip- Ronald G Sommer- US patent 5,569,608 (Oct 29, 1996)

6 Horses Crab amoebocyte lysate factor G activation inhibitor- Tanaka S.- US patent 5,641,643 (Jun, 24, 1997)

7 Reagent for assaying endotoxin. Tanak S- US patent 5,605,806 (Feb 25, 1997)

8 Test articles for performing dry reagent prothrombin time assays Stephen E. Zweig et al. US patent 5,418,141 (May 23, 1995)

9 Composite cellulose nitrate membrane on polyester support- Beer et al- US patent 5,628,960 (May 13, 1997)

10 One step-test device. Philip W. Sayles. US patent 5,591,401 (Jan 7, 1997)

CLAIMS

1. Solid phase apparatus, for detection of endotoxin in a liquid sample using reagents located within solid support material, comprising the reagents:-

endotoxin sensitive reagent; and

buffer components;

wherein when a liquid sample containing endotoxin is applied to the apparatus the endotoxin sensitive reagent reacts with the endotoxin to form at least one product, and following contact of the at least one product with buffer components an endotoxin indicator is formed.

2. Solid phase apparatus according to Claim 1 further comprising a colour developer, which reacts with the endotoxin indicator to form a coloured product.

3. Solid phase apparatus according to Claim 1 or 2 wherein the reagents are differentially located within portions of the solid support material, and wherein the apparatus comprises:-

an application portion to receive a liquid sample, and containing endotoxin sensitive reagents comprising endotoxin sensitive factor and a first chromogenic substrate, and being in communication with a buffer portion;

the buffer portion containing buffer components, and being in communication with an indicator portion; and

the indicator portion optionally containing a colour developer or being adapted to receive application of a colour developer by a user

wherein in use when a liquid sample containing endotoxin is applied to the application portion, endotoxin reacts with endotoxin sensitive factor to form a product which in turn reacts with the first chromogenic substrate to form a second chromogenic substrate, and contact of the second chromogenic substrate with buffer components forms an endotoxin indicator.

4. Solid phase apparatus according to Claim 3 wherein the application portion comprises:-

a first portion containing an endotoxin sensitive factor, in communication with

a second portion containing a first chromogenic substrate, in communication with the buffer portion.

5. Solid phase apparatus according to Claim 3 or 4 wherein the buffer portion comprises:-

a third portion containing first buffer components, in communication with

a fourth portion containing second buffer components, in communication with the indicator portion, whereby in use contact of the second chromogenic substrate with the first and then the second buffer components results in formation of the endotoxin indicator.

6. Solid phase apparatus according to Claim 3, 4 or 5 wherein the indicator portion is substantially free of solute capable of altering pH.

7. Solid phase apparatus according to any of Claims 1-6 for the detection of endotoxin in a liquid sample comprising a solid support in the form of a test dipstick, a flat sheet or a cylindrical capillary tube wherein said solid support is selected from chromatographic papers, silica nitrocellulose, hydrophillic polymer, dextran, and glass fibres.

8. Solid phase apparatus according to any previous claim in the form of a said test strip which comprises several portions made with hydrophillic matrix from which ß-glucans have previously been extracted with a solvent system;

9. Solid phase apparatus according to Claim 8 wherein the test strip is constructed in a flat sheet form, said portions are constructed in a plane form to permit capillary flow, between respective portions.

10. Solid phase apparatus according to any previous claim wherein the solid support material is mounted in a closed and pyrogen free transparent casting module to achieve pyrogen free manipulation.

11. Solid phase apparatus according to Claim 10, wherein the said transparent casting module can be opened at the bottom to permit contact with the sample or can be placed in a sample reservoir and can be opened at the top to permit contact with chemical reactants.

12. Solid phase apparatus according to any previous claim wherein the colour developer is N1-napthylethylenediamine.

13. Solid phase apparatus according to any previous claim wherein the endotoxin sensitive factor a limulus amoebocyte lysate or other factors which react with endotoxin to produce coagulin.

14. Solid phase apparatus according to any previous claim wherein the first chromogenic substrate is t-butoxycarbonyl-leucyl-glycyl-arginine-paranitroanilide (Boc-Leu-Gly-Arg-pNA) and paranitroanilide is the second chromogenic substrate.

15. Solid phase apparatus according to Claim 14, wherein the endotoxin indicator is p-nitroaniline.

16. Solid phase apparatus according to any previous claim wherein the buffer components contain hydrochloride and sodium nitrite ions.

17. Solid phase apparatus according to Claim 16, wherein the buffer components contain hydrochloride and ammonium ions.

18. Solid phase apparatus according to any previous claim wherein the indicator portion is a neutral portion.

19. Solid phase apparatus according to any previous claim wherein contact on the second chromogenic substrate with buffer components converts it to a diazonium salt.

20. Solid phase apparatus according to Claim 19, wherein which p-nitroaniline is derivatized to N1-napthylethylenediamine.

21. Solid phase apparatus according to Claim 20, wherein the indicator portion is changed to a highly visible red colour when a drop of a solution containing N1-naphtylethylenediamine is added to form a diazoamino of a highly visible red colour.

22. Solid phase apparatus according to Claim 20, wherein the indicator portion contains a solid support and N1-naphtylethylenediamine to form a diazoamino of a highly visible red colour.

23. A method of assaying for the presence of endotoxin in a liquid sample, comprising

combining a test amount of the sample with reagents in a first region of solid phase support material to form at least one product;

allowing the one or more products to flow through a second region of solid support material containing buffer components thereby to convert the at least one product into endotoxin indicator; and

testing for the presence of the endotoxin indicator.

24. A method according to Claim 23 wherein endotoxin indicator is coloured.

25. A method according to Claim 23 further comprising reacting, in a region of solid support material, the endotoxin indicator with a colour developer to form a coloured product.

26. A method according to any of Claims 23 to 25 comprising heating the support material to activate the reagents.

27. A method according to any of Claims 23 to 26 comprising measuring the intensity of the coloration of the endotoxin indicator to determine concentration of endotoxin.

28. A kit for testing for the presence of endotoxin in a liquid sample, comprising at least two solid phase apparatus according to any of claims 1 to 22, wherein the at least two apparatus have different sensitivities to endotoxin.

29. A kit according to Claim 28 comprising a plurality of solid phase apparatus of different sensitivities to endotoxin for quantitative determination of endotoxin in a liquid sample.

30. Use of solid phase material containing buffer components in manufacture of solid phase apparatus for testing for the presence of endotoxin in a liquid sample.

31. Use of solid phase material containing endotoxin sensitive reagent in manufacture of solid phase apparatus for testing for the presence of endotoxin in a liquid sample.

FIG. 2
PAPER TEST STRIP

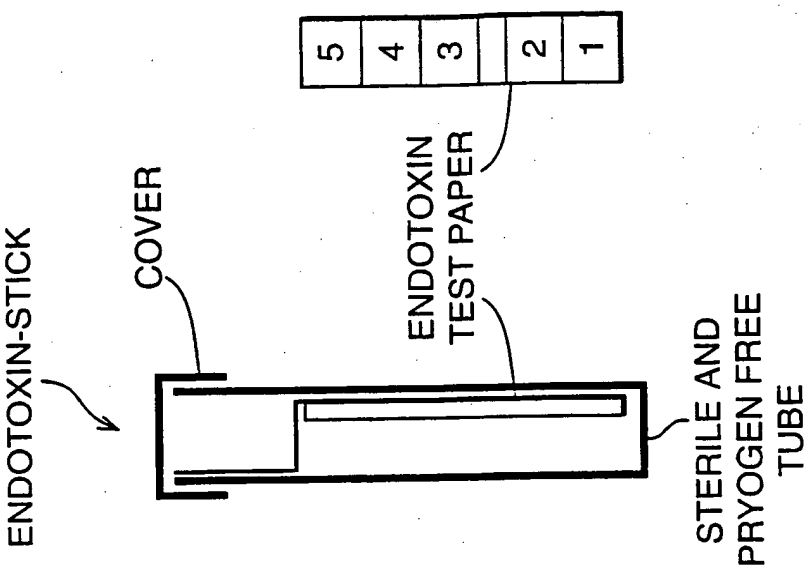


FIG. 1
SCHEMATIC REPRESENTATION OF THE CAPILLARY TEST STRIP

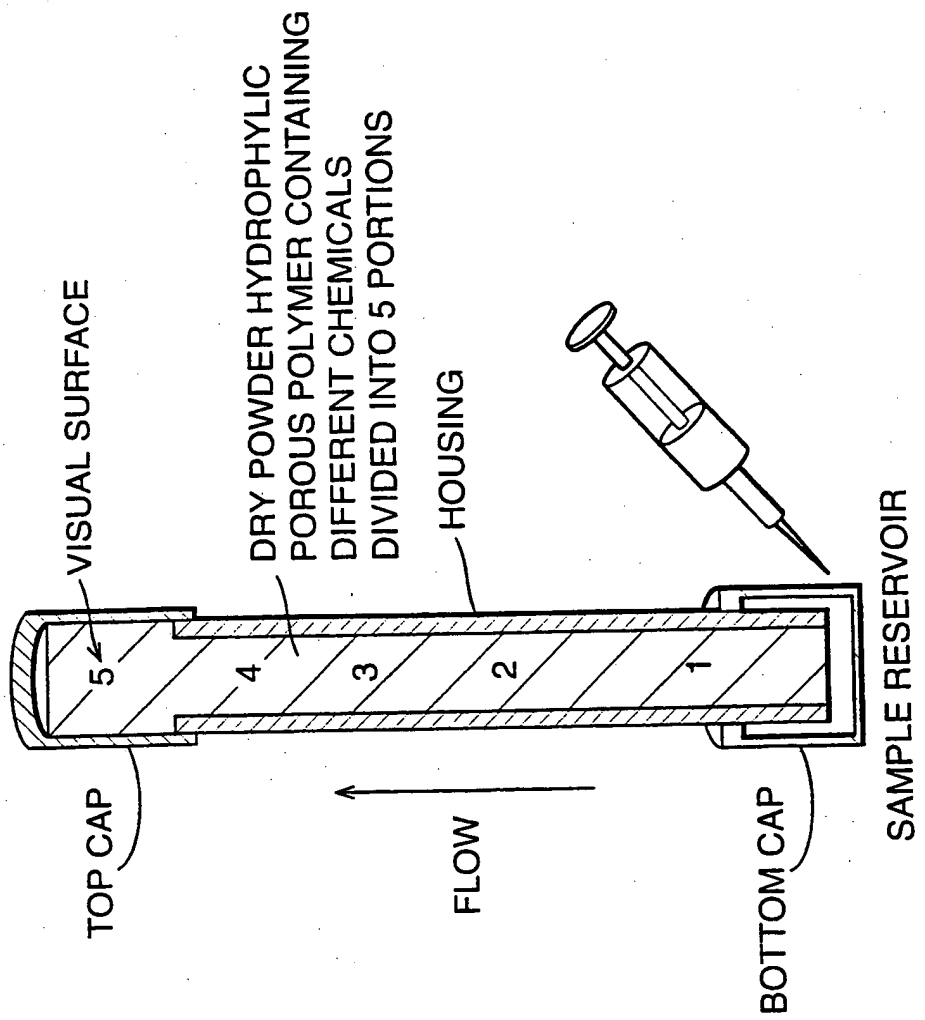
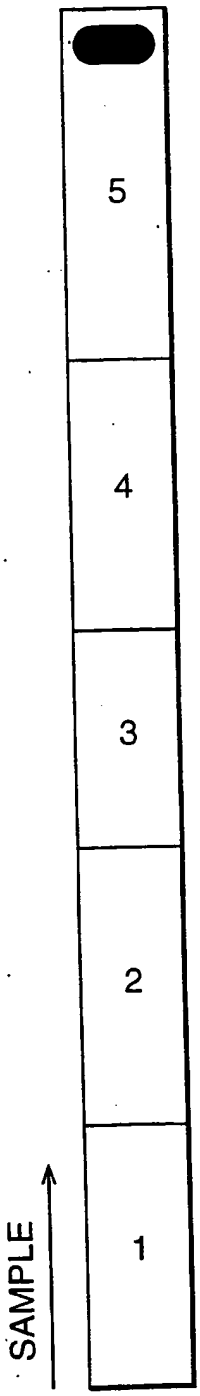


FIG. 3

TEST STRIP REACTION PROCEDURE



N1-NAPTHYLENDIAMINE → RED → DIAZOAMINO

P-NITROANILINE —DERIVATIZATION→ DIAZONIUM

SUBSTRATE —ENZYME→ P-NITROANILINE

PROENZYME —ENDOTOXIN→ COAGULOGEN CLEAVAGE PRODUCT

ENZYME

FIG. 4

SENSITIVITY OF LPS-CHROMOGENIC SUBSTRATE BY DIFFERENT LYSATE ACTIVITIES

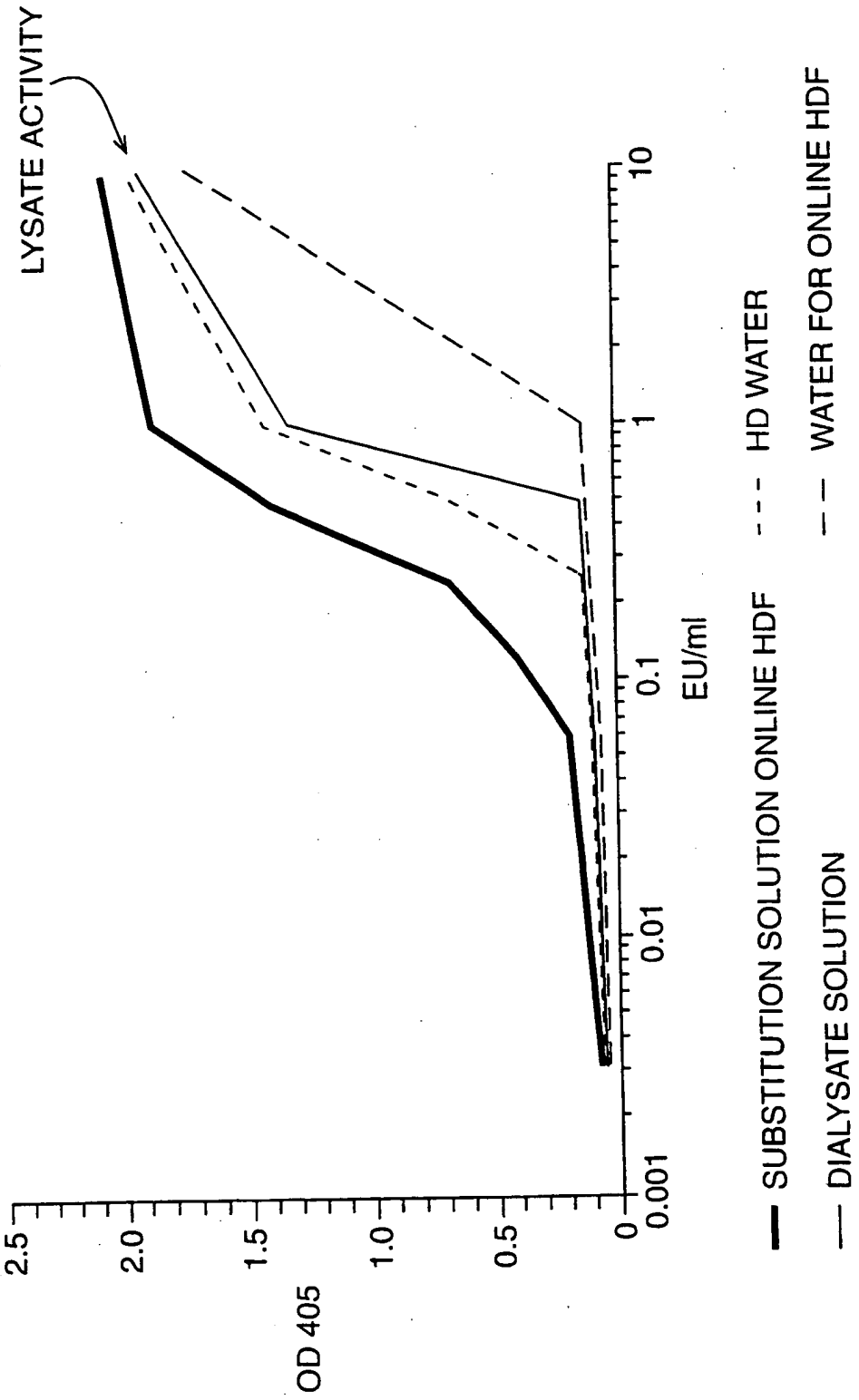
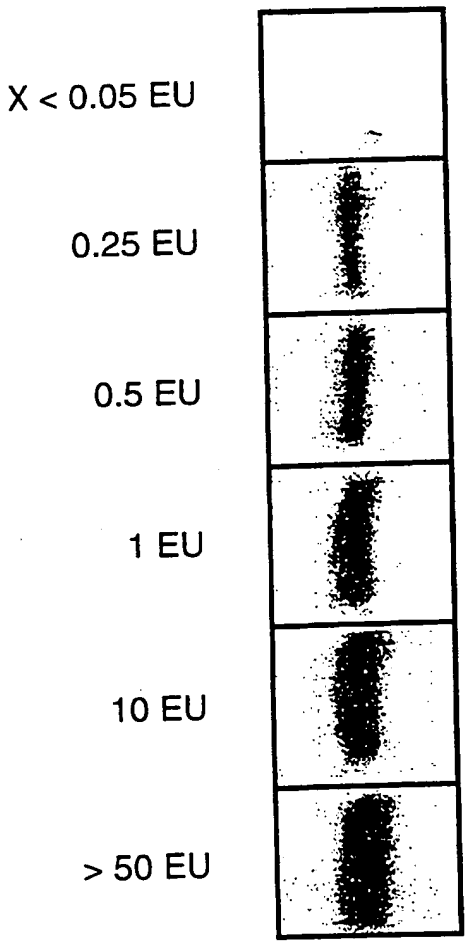


FIG. 5
DETECTION SPOTS



INTERNATIONAL SEARCH REPORT

Interi ·ial Application No
PCT/GB 99/01099

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/579 G01N33/569 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 717 658 A (MICHAELS ANGELA A) 5 January 1988 (1988-01-05) column 5, line 61 - column 6, line 44 column 8, line 56 - line 62 ---	1,2,23, 30,31
X	EP 0 588 303 A (SEIKAGAKU KOGYO CO LTD) 23 March 1994 (1994-03-23) cited in the application the whole document ---	1,2,23, 30,31
A	GUILFOYLE D E ET AL: "Evaluation of a chromogenic procedure for use with the Limulus lysate assay of bacterial endotoxins in drug products." JOURNAL OF PARENTERAL SCIENCE AND TECHNOLOGY, (1985 NOV-DEC) 39 (6) 233-6 , XP002110748 the whole document --- -/--	1,2,23, 30,31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 August 1999	17/08/1999

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 99/01099

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 591 645 A (ROSENSTEIN ROBERT W) 7 January 1997 (1997-01-07) cited in the application abstract	1,23,30, 31
A	EP 0 265 127 A (HARVEY WILSON ;WILSON MICHAEL (GB)) 27 April 1988 (1988-04-27) examples	1,23,30, 31
A	EP 0 291 856 A (BAEK LEIF ;KOCH CLAUS (DK)) 23 November 1988 (1988-11-23) examples	1,23,30, 31
A	US 5 356 778 A (HANSEN ERIC J ET AL) 18 October 1994 (1994-10-18) abstract	1,23,30, 31
A	WO 97 44665 A (NEELY CONSTANCE F ;UNIV PENNSYLVANIA (US)) 27 November 1997 (1997-11-27) the whole document	1,23,30, 31

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/GB 99/01099

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4717658 A	05-01-1988	AT 60136 T	15-02-1991
		AU 576102 B	11-08-1988
		AU 6583086 A	04-06-1987
		CA 1292175 A	19-11-1991
		DK 114692 A	17-09-1992
		DK 579686 A	04-06-1987
		EP 0224830 A	10-06-1987
		FI 864890 A,B,	04-06-1987
		IE 59008 B	15-12-1993
		JP 6341993 A	13-12-1994
		JP 1916125 C	23-03-1995
		JP 6048272 B	22-06-1994
		JP 62134563 A	17-06-1987
		NO 173744 C	26-01-1994
EP 0588303 A	23-03-1994	JP 6130064 A	13-05-1994
		AU 678173 B	22-05-1997
		CA 2106119 A	15-03-1994
		CN 1091832 A	07-09-1994
		US 5550030 A	27-08-1996
US 5591645 A	07-01-1997	AT 123575 T	15-06-1995
		AU 605565 B	17-01-1991
		AU 1359588 A	29-09-1988
		CA 1303983 A	23-06-1992
		DE 3853927 D	13-07-1995
		DE 3853927 T	16-11-1995
		DK 168188 A	28-09-1988
		EP 0284232 A	28-09-1988
		ES 2074994 T	01-10-1995
		FI 880764 A,B	28-09-1988
		GR 3017177 T	30-11-1995
		JP 1063865 A	09-03-1989
		JP 7013640 B	15-02-1995
		JP 10073592 A	17-03-1998
EP 0265127 A	27-04-1988	WO 8802862 A	21-04-1988
		GB 2197470 A,B	18-05-1988
		JP 2500856 T	22-03-1990
EP 0291856 A	23-11-1988	AT 116444 T	15-01-1995
		AU 611456 B	13-06-1991
		AU 1943488 A	21-12-1988
		CA 1340436 A	16-03-1999
		CN 1030138 A,B	04-01-1989
		DE 3852568 D	09-02-1995
		DE 3852568 T	03-08-1995
		DK 576789 A,B,	17-11-1989
		WO 8809507 A	01-12-1988
		ES 2069535 T	16-05-1995
		FI 95627 B	15-11-1995
		JP 2504308 T	06-12-1990
		JP 2667695 B	27-10-1997
		PT 87524 A,B	31-05-1989
		US 5591628 A	07-01-1997
		US 5316911 A	31-05-1994
US 5356778 A	18-10-1994	US 5198339 A	30-03-1993

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/GB 99/01099

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5356778 A		DE 69110287 D	13-07-1995
		DE 69110287 T	08-02-1996
		EP 0539497 A	05-05-1993
		AT 123576 T	15-06-1995
		AU 8297191 A	04-02-1992
		CA 2084083 A	14-01-1992
		WO 9201228 A	23-01-1992
WO 9744665 A	27-11-1997	US 5773306 A	30-06-1998
		AU 3140497 A	09-12-1997
		EP 0906574 A	07-04-1999